

Population genetic structure and diversity of high value vulnerable medicinal plant *Acorus calamus* in India using RAPD and chloroplast microsatellite markers

H. S. Ginwal • Neha Mittal • Arvind Tomar • V. K. Varshney

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Abstract: *Acorus calamus* is a highly valued medicinal plant with global distribution used in several drugs of health care systems. We evaluated the genetic diversity and population structure of 50 populations of *A. calamus* from different geographical regions in India through RAPD and chloroplast microsatellite markers. From the total screened 82 RAPD primers and 18 cpSSR primers, 10 RAPD and nine cpSSRs were found polymorphic. The selected 10 RAPD primers produced a total of 96 reproducible bands, out of which 65 were polymorphic (67.70%). Whereas, the selected nine cpSSR markers produced 26 alleles and all of them were polymorphic. The mean genetic diversity (H_T) among populations using RAPD ($H_T = 0.263$) and cpSSR ($H_T = 0.530$) markers was higher in comparison to the mean genetic diversity within populations. Mean coefficient of gene differentiation (G_{ST}) between the populations was also high for both RAPD ($G_{ST} = 0.830$) and cpSSR markers ($G_{ST} = 0.735$), whereas the estimated gene flow was very low for RAPD ($N_m = 0.102$) and for cpSSR ($N_m = 0.179$). AMOVA analysis revealed that more genetic variation resided among the populations than within populations. Significant differences ($p < 0.001$) were observed between the populations and individuals within the populations. Cluster analysis of RAPD and cpSSR data using UPGMA algorithm based on Nei's genetic similarity matrix placed the 50 populations into two main clusters. The implication of the results of this study in devising strategy for conservation of *A. calamus* is discussed.

Keywords: *Acorus calamus*; cpSSR; RAPD; population structure; genetic diversity

Introduction

The Indian subcontinent has a rich repository of medicinal plants that are used by various indigenous health care systems. As per the estimate, over 7000 species of medicinal plants are used for medicinal purposes (Uma Shaanker and Ganeshaiah 1997). However, due to an indiscriminate use of these resources over time and fragmentation of habitats, many of these species are increasingly threatened and face the risk of being genetically impoverished (Uma Shaanker and Ganeshaiah 1997).

Acorus calamus (Sweetflag) is an ethnobotanically important medicinal and aromatic plant with a global distribution (Pai 2005). Its ubiquitous presence in several tropical and temperate regions is attributed to the intentional introduction of its rhizome by humans (Pai 2005). In India, it is used in several drugs of the Unani and Ayurvedic health care systems (Ravindran and Balachandran 2004). The essential oil obtained from the rhizome called calamus oil contains 'β-Asarone', which is carcinogenic in nature (Bertea et al. 2005). Its concentration in calamus oil generally depends on the ploidy level of the plant, which varies with the geographical distribution/occurrence of the species (Ogra et al. 2009). Based on ploidy status and geographical distribution, *A. calamus* has been classified as (i) diploid ($2n = 2x = 24$; North America), (ii) triploid ($2n = 3x = 36$; Europe), (iii) the tetraploid ($2n = 4x = 48$; East Asia, India and Japan) and (iv) hexaploid ($2n = 6x = 72$; Kashmir area) (Ogra et al. 2009).

A. calamus grows in varying agro climatic conditions in India right from tropical to temperate marshes from Kashmir to the north east ascending to an altitude of 1,500–2,200 m in the Himalayan ranges (Ogra et al. 2009). This species inhabits perpetually wet areas like the edges of streams and around ponds and lakes, in ditches and seeps. It is often found growing close to the sites of Indian villages, camping areas or trails. Plants of *A.*

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H. S. Ginwal (✉) • Neha Mittal

Division of Genetics and Tree Propagation, Forest Research Institute, Dehradun, Uttarakhand 248195, India. Email: ginwalhs@icfre.org; ginwalhs@rediffmail.com

Arvind Tomar • V. K. Varshney

Chemistry of Forest Products Division, Forest Research Institute, Dehradun, Uttarakhand 248195 (India)

Responsible editor: Hu Yanbo

calamus very rarely flower or set fruits and if flowers, it takes from early to late summer depending on the latitude. Plant is mainly insect pollinated and the gene flow through pollens is limited within shorter distances. The seed dispersion of the species occurred by means of wind (Buzgo and Endress 2000).

The species has been categorized as a highly prioritized medicinal plant as it has immense value in curing various diseases (Kala et al. 2004). However, it is in the low niche and is in the freeway towards extinction due to over exploitation, habitat disturbance (Barbhutiya et al. 2009) and un-favored reproductive system. Due to these factors, the wild populations of this species are declining sharply in India. As a consequence, in small isolated populations, inbreeding may increase; genetic drift tends to predominate over mutation, selection and migration, making them prone to lose adaptive genetic diversity (Yonghua et al. 2010). Ultimately, the species may risk extinction due to loss of genetic diversity (Wright 1943; Frankham et al. 2002).

A. calamus has been considered vulnerable and threatened species in India (Kala et al. 2004). A viable conservation strategy is needed for preserving the dwindling genetic resources of this species. The determination of population genetic structure is of great importance in the development of strategies for collecting and conserving plant materials as genetic resources, as well as in improvement for their utilization. Being under biotic and abiotic stresses, it is feared that genetic diversity of *A. calamus* populations in India may decrease. Hence, as a prerequisite the amount of genetic diversity within and among populations need to be investigated to guide strategies for their conservation and sustainable utilization.

DNA based markers being neutral and not influenced by the environmental conditions help in providing reliable information on the characteristics of the genetic material (Subramanyam et al. 2009). Keeping above in view, the present study describes molecular characterization and genetic diversity estimation among and within populations of *A. calamus* collected from different geographical regions of India using random amplified polymorphic DNA (RAPD) and chloroplast microsatellite markers (cpSSR). Specifically, our goals were to (1) estimate the level and distribution of genetic diversity among and within populations from various geographical locations of India, (2) develop population genetic structure and estimate genetic differentiations between the populations, and (3) estimate the gene flow among populations.

Materials and methods

Plant material

Foliage of *A. calamus* was collected from 50 geographically distinct small populations (about 5–20 m² each) in India. Ten plants were randomly sampled from each population for the collection of leaves. The plants considered for collection were well spaced and separated from each other by at least 5–10 m. Samples were brought to the laboratory and stored at -80°C. The detail regarding the geographical locations of the populations is

given in Table 1.

DNA extraction and RAPD analysis

Genomic DNA was extracted from freshly collected leaves using the CTAB miniprep protocol developed by Ginwal and Mittal (2010). Eight Operon RAPD primers (QIAGEN Operon, 1000 Atlantic Avenue, Alameda, CA 94501, USA) viz. OPBH-05, OPBH-19, OPAG-19, OPBG-03, OPA-11, OPA-12, OPA-15, OPG-09, and two primers given by Mosselers et al. (1992) i.e.: M-122 and M-119 (Table 2) were selected from the screened 82 random primers based on their ability to detect distinct polymorphic and reproducible amplified products across the populations. Amplification with each primer was repeated at least twice in order to ensure reproducibility and only those bands, which occurred consistently, were considered for scoring and analysis. The reactions were carried out in a Mastercycler gradient PCR system (Eppendorf Pvt Ltd, Germany). Each 25 µl reaction volume contained about 10 ng template DNA, 1X PCR reaction buffer (100 mM Tris pH 9.0, 500 mM KCl, 0.1% gelatin), 2.0 mM MgCl₂ (GeNeiTM, Bangalore, India), 0.2 mM dNTPs (GeNeiTM, Bangalore, India), 0.4 µM of single primer and 1 U of *Taq* DNA polymerase (GeNeiTM, Bangalore, India). The reaction master mix for 25 µl PCR was prepared as 1.0 µl template DNA, 2.5 µl of 10 X assay buffer, 2 µl of MgCl₂, 2 µl dNTPs, 0.2 µl of *Taq* DNA polymerase and 16.8 µl sterile millipore water. The thermal cycler was programmed for an initial denaturation step of 3.5 min at 94°C, followed by 44 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 37°C; extension was carried out at 72°C for 2 min and final extension at 72°C for 7 min and a hold temperature of 4°C at the end. Control samples containing all reaction reagents except DNA were evaluated to verify the presence of contaminants.

Amplified RAPD fragments were separated electrophoretically on 1.5% agarose gel in 1X TBE buffer, stained with ethidium bromide (0.5 µg/ml) and photographed with the gel documentation imaging system (UVP Model LMS-20E, Upland, USA). DNA from each individual was amplified with the same primer more than once to test its reproducibility. The banding patterns were compared with ϕ X 174 DNA/*Bsu*RI (*Hae*III), which was used as size marker (GeNeiTM, Bangalore, India) to know the size of the amplified DNA fragments.

Chloroplast microsatellite marker analysis

A total nine polymorphic chloroplast microsatellite markers (Table 3) developed by Ginwal et al. (2009) were used for the present study. The reactions were carried out in a Mastercycler gradient PCR machine (Eppendorf Pvt Ltd, Germany). Each 25 µl reaction volume contained 10 ng of DNA samples, 2.5 µl of 1X reaction buffer (100 mM Tris pH 9.0; 500 mM KCl; 0.1% gelatin), 2.5 mM MgCl₂, 200 µM (0.2 mM) of each dNTPs (GeNeiTM, Bangalore, India), 0.2 µM of each forward and reverse primer (Operon Biotechnologies, Germany) and 1 unit of *Taq* DNA polymerase (GeNeiTM, Bangalore, India). The polymerase chain reaction protocol used the 5 min denaturation at 94°C; then sam-

ples were incubated for 35 cycles of denaturation at 94°C for 30s, optimized annealing at T_m for 1 min (Table 3) and extension at 72°C for 2 min. The reactions were completed by incubating the

samples at final extension for 10 min at 72°C. Control samples containing all reaction reagents except DNA were evaluated to verify the presence of contaminants.

Table 1. Geographical attributes of the collected populations of *A. calamus*

Population Index	Origin		Latitude (°N)	Longitude (°E)	Altitude (masl)
	State	Locality			
A-1	Uttarakhand	FRI Campus (Dehradun)	30.19	78.04	635
A-3	Uttar Pradesh	NBRI Campus (Lucknow)	26.55	80.59	123
A-4	Harayana	Hissar	29.1	75.43	211
A-9	Himachal Pradesh	Solan (Shilly)	30.92	77.12	1467
A-10	Uttarakhand	Dhanaulti	30.19	78.04	635
A-13	Uttarakhand	Gullarghati (Dehradun)	30.19	78.04	635
A-14	Uttarakhand	Mandi (karsog)	31.13	76.37	754
A-16	Uttarakhand	Almora	29.36	79.3	1646
A-17	Jammu	Parol (Nagri, Kathua)	32.17	75.32	218
A-18	Jammu	Jandi, Kathua (Hiranagar)	32.27	75.16	307
A-19	Jammu	Khokharchak, (Ramgarh, Sambha)	32.56	75.11	384
A-20	Jammu	Baghajanha,	32.44	74.52	305
A-21	Jammu	Beril, Kathua, Teh:- Billawar	32.17	75.32	218
A-22	Uttarakhand	Chamba (Chamni)	30.22	78.28	1676
A-23	Uttarakhand	Maneri (Didsari, Uttarkashi)	30.75	78.31	1537
A-24	Uttarakhand	Gangori	30.73	78.45	1558
A-25	Uttarakhand	Srikot (Khanda, Shrinagar)	30.11	78.47	545
A-26	Uttarakhand	Devar (Pokhari, Chamoli)	30.19	79.11	1738
A-27	Uttarakhand	Mandal (Khalla, Chamoli)	30.26	79.16	1588
A-28	Manipur	Mao Gate Area	23.83	93.03	790
A-29	Uttarakhand	Khunigad (Uttarkashi)	30.58	77.58	1135
A-30	Uttarakhand	Naugaon (Uttarkashi)	30.47	78.07	1046
A-32	Himachal Pradesh	Sirmour (Panjola, Kotla)	30.51	77.8	1190
A-33	Himachal Pradesh	Solan (Khaltoo)	30.51	77.1	939
A-34	Uttarakhand	Bhagwati (Almora)	29.51	79.18	878
A-35	Uttarakhand	Chokhutia (Ganai, Almora)	99.52	79.21	954
A-36	Uttarakhand	Agarchatti (Ramratalla, Chamoli) Dwarahat (Almora)	30	79.18	1321
A-37	Uttarakhand	Soni (Almora)	29.45	79.25	1399
A-38	Uttarakhand	Khargot (Pithoragarh)	29.37	79.21	1636
A-39	Uttarakhand	Lohaghat (Champawat)	29.33	80.11	1331
A-40	Uttarakhand	Champawat (Champawat)	29.24	80.5	1565
A-41	Uttarakhand	Jatroon (Chamba)	29.19	80.5	1597
A-42	Himachal Pradesh	Mundi (Salah, Chamba)	32.25	75.59	916
A-43	Himachal Pradesh	Khaziar lake (Chamba)	32.21	76	1020
A-44	Himachal Pradesh	Olie (Chamba)	32.32	76.03	1920
A-45	Himachal Pradesh	Sultanpur (Fish farm, Chamba)	32.32	76.07	1074
A-46	Himachal Pradesh	Sihunta (Ghanota, Chamba)	32.33	76.6	841
A-47	Himachal Pradesh	Rait (Kangra)	32.18	76.5	874
A-48	Himachal Pradesh	Kachhred (Mumta, Kangra)	32.11	76.13	669
A-49	Himachal Pradesh	Dhanhadi (Una, Tikarla)	32.05	76.21	794
A-50	Himachal Pradesh	Check-i-Kawoosa (Narbae, Budgam)	31.36	76.08	430
A-51	Jammu and Kashmir	Hokersar (Budgam)	34.07	74.38	1604
A-52	Jammu and Kashmir	Jarmola Parola,(Uttarkashi)	34.06	74.39	1570
A-53	Uttarakhand	Kateshwar, Jageshwar (Almora)	30.56	78.05	1842
A-54	Uttarakhand	Digoli, Barichhina (Almora)	29.37	79.51	1767
A-55	Uttarakhand	Mori, Teh:- Mori, District:-Uttarkashi	29.38	79.43	1333
A-56	Uttarakhand	Gohalkun, District:- Baramulla	41	78.03	1236
A-57	Jammu and Kashmir	Bajwudar, District:- Srinagar	34.14	74.3	1608
A-58	Jammu and Kashmir	Sangam (Anchar) District:- Srinagar	34.08	74.47	1570
A-59	Jammu and Kashmir		34.08	74.47	1580

Table 2. RAPD primers used for population genetic diversity analysis in *A. calamus*

Primer	Nucleotide Sequence (5'-3')	Total No. of scorable bands	No. of polymorphic bands	Polymorphism (%)	Size range (bp)
OPBH-05	GTA GGT CGC A	12	12	100.00	1,353 -194
OPBH-19	GTC GTG CGG A	10	5	50.00	1,353 -281
OPAG-19	AGC CTC GGT T	10	10	100.00	1,353 -194
OPBG-03	GTG CCA CTT C	6	4	66.66	1,353 -872
OPA-11	CAA TCG CCG T	10	6	60.00	1,353 -310
OPA-12	TCG GCG ATA G	7	5	70.14	1,353 -310
OPA-15	TTC CGA ACC C	10	8	80.00	1,353-310
OPG-09	CTG ACG TCA C	10	3	30.00	1,353 -310
M-122	GTA GAC GAG C	12	7	58.33	1,353 -310
M-119	ATT GGG CGA T	9	5	55.55	1,353 -310
Maximum		12	12	100.00	1,353
Minimum		6	3	30.00	194
Total		96	65	67.70	

Table 3. Repeat motif, forward and reverse primer sequences, annealing temperature and expected product size of cpSSR markers used for population genetic diversity analysis in sweet flag (*A. calamus*)

Marker name	Repeat type	Repeat Motif	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
AC-01	Pentanucleotide	(GAAGG) ₃	tacgttctctttatggacc	attattgatcgatttgacg	56	271
AC-03	Tetranucleotide	(AAGC) ₃	aaggtttacattggacgaaa	acaaccagaaagcagaaggta	56	337
AC-04	Trinucleotide	(ATT) ₄	agaaatcagtggaattcatgg	attcgaacaagaagaacgaa	54	274
AC-05	Dinucleotide	(AT) ₇	actattccctcccgtatgtt	gaaccaatccaattaatcca	53	188
AC-06	Pentanucleotide	(AATAA) ₃	ttacaatgcgatgctctaa	ggaatcctgctctgctataa	56	373
AC-07	Pentanucleotide	(TTTATT) ₄	cgatggataagaatcctgag	ttcatatgtatgacgcaacc	53	382
AC-08	Pentanucleotide	(AAGGG) ₃	aaggattgagccgaataaa	aagtttctcttgcataacgg	53	325
AC-14	Pentanucleotide	(ATTAA) ₃	atctttcacattcgctaga	ccgctgcattcttattatt	54	315
AC-15	Trinucleotide	(TAA) ₄	cgggaataagcgagataaatg	gccatattcggtatctgaag	56	385

Statistical analysis

RAPDs across 50 populations (500 plants) were visually scored for the presence (1) or absence (0) for each amplified fragment. Data spreadsheet was prepared with binary data for the genetic analysis. Frequencies of the RAPD bands were used to calculate the level of polymorphism. The binary data of RAPD markers so generated were used to estimate genetic parameters (total gene diversity - H_T , diversity within population - H_S and gene flow - N_m) as a diploid dominant marker system using POPGENE program version 1.32 (Yeh et al. 1999).

For cpSSR markers, because of the non-recombining nature of the chloroplast genome, each chloroplast primer pair was consider as 'locus' to refer to a cpSSR site (defined by the termini of a PCR primer pair) and length variants at a cpSSR site were treated as 'alleles'. The cpSSR data were visually scored by the presence of specific alleles in each individual of 50 populations at a SSR locus. The scored cpSSR data was analyzed as a haploid co-dominant marker using POPGENE program version 1.32 (Yeh et al. 1999) to estimate the total gene diversity (H_T), diversity within population (H_S), and gene flow (N_m) (as per Nei 1978). The amount of genetic differentiation among populations was estimated using the G_{ST} measure (as per Nei 1987). The

simple correlations between the geographical coordinates (latitude, longitude and altitude) and gene diversity of populations were estimated, as per the method described by Hodge et al. (2002). The Nei's similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean averages (UPGMA) and a dendrogram was also generated using the same software. The levels of differentiation among the populations was estimated from Analysis of Molecular Variance (AMOVA), which was carried out according to the method of Excoffier et al. (1992) using the Arlequin software version 3.11 (Excoffier et al. 2005). In the AMOVA analysis, the sources of variation were divided into two nested levels: among populations and among individuals within populations.

Results

Genetic diversity with RAPD markers

The largest fragments amplified from the various RAPD primer pairs were found in the range of 1353 bp to 1078 bp while the smallest but easily recognizable fragments were found approximately in the range of 234–194 bp. Most of the fragments were

concentrated between 1,353 bp and 194 bp. A total of 96 bands were detected from 10 RAPD primers, out of which, OPBH-05 and M-122 amplified the maximum number of bands (12) and the primer OPBG-03 amplified the minimum number of bands (6) in compared to the others primers. The number of bands scored for each primer varied from 6 to 12 with an average of 9.6 per primer.

Out of the total 96 scored bands, 65 bands were found polymorphic (67.70%) and 31 monomorphic (32.30%), revealing a high degree of polymorphism. Per cent polymorphism was the highest (100%) for the primer OPBH-05 and OPAG-19 and the lowest (30%) for the primer OPG-09 (Table 2). Averaged polymorphism per primer and per population was 65% and 10.64%, respectively. The RAPD pattern with primer OPAG-19 is shown in Fig. 1.

The most informative RAPD primers were OPA-11 and OPA-15, which amplified bands of 0.6 kb and 0.87 kb, respectively, that were present only in the populations A-44, A-51, A-52, A-57, A-58 and A-59, and absent in all other populations. Likewise, OPBH-19 and M-119 primer amplified a band <0.3 kb, present only in the populations A-44, A-51, A-52, A-57, A-58 and A-59 and absent in all the other populations. The primer OPG-09 amplified a band <0.6 kb, present in all the populations except A-44, A-57, A-58 and A-59. Among the various bands amplified by the primer M-122, two fragments with the band size of 1 kb and 0.8 kb were very specific to only one population i.e. A-44.

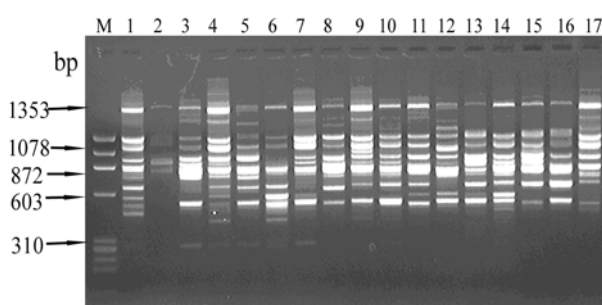


Fig. 1 RAPD profile of *A. calamus* genotypes (Lane 1–17) generated using random decamer primer OPAG-19. Photograph showing the amplified polymorphic bands. Lane M is the molecular weight marker- ϕ X-174 DNA/*Bsu*RI (*Hae*III).

The averaged total gene diversity (H_T) among the populations was higher (0.263 ± 0.026) than that within a population ($H_S = 0.044 \pm 0.002$). Maximum gene diversity was found within the population A-45 (0.134) and minimum in A-59 (0.010). The mean coefficient of population differentiation (G_{ST}) among the various populations was 0.830, showing that 17% of the variation was present within populations. The estimated gene flow (N_m) between the populations was low (0.102) (Table 4). The genetic identity based on the Nei's genetic similarity index ranged from 0.49 to 0.98. The highest genetic similarity (0.98) was found between the populations A-17 and A-18, while the lowest genetic similarity (0.49) was found between populations

A-39 & A-57 and A-39 & A-59, indicating high genetic divergence between them.

Table 4. Genetic variability across all the populations of *A. calamus* using RAPD and cpSSR markers

Primers	Observed number of alleles	Effective number of alleles	H_T	H_S	G_{ST}	Gene flow (N_m)
RAPD markers						
M-122	1.75	1.420	0.226	0.009	0.945	0.034
M-119	2.00	1.227	0.180	0.032	0.738	0.132
OPG-09	2.00	1.226	0.170	0.040	0.686	0.295
OPA-11	2.00	1.427	0.257	0.029	0.793	0.175
OPA-12	2.00	1.388	0.220	0.030	0.653	0.202
OPA-15	2.00	1.307	0.220	0.019	0.857	0.114
OPBG-03	2.50	1.391	0.260	0.038	0.709	0.444
OPBH-05	2.00	1.603	0.355	0.044	0.869	0.077
OPBH-19	2.00	1.479	0.295	0.061	0.743	0.192
OPAG-19	2.00	1.736	0.456	0.131	0.678	0.253
Maximum	1.75	1.736	0.456	0.131	0.945	0.444
Minimum	2.50	1.226	0.170	0.009	0.653	0.034
Mean	1.947	1.428	0.263	0.044	0.830	0.102
	(0.223)	(0.332)	(0.026)	(0.002)		
cpSSR markers						
AC-01	3.00	2.510	0.603	0.143	0.762	0.155
AC-03	2.00	1.991	0.498	0.067	0.865	0.078
AC-04	3.00	2.245	0.561	0.108	0.806	0.120
AC-05	3.00	2.229	0.549	0.188	0.656	0.261
AC-06	3.00	2.048	0.512	0.192	0.624	0.300
AC-07	3.00	2.285	0.567	0.155	0.726	0.188
AC-08	3.00	1.622	0.382	0.083	0.782	0.138
AC-14	3.00	2.740	0.633	0.182	0.712	0.202
AC-15	3.00	1.878	0.463	0.140	0.696	0.217
Maximum	3.00	2.510	0.633	0.192	0.865	0.300
Minimum	2.00	1.622	0.382	0.083	0.624	0.078
Mean	2.88	2.172	0.530	0.140	0.735	0.179
	(0.33)	(0.334)	(0.005)	(0.002)		

* The values in the brackets are standard deviation. H_T ---Total gene diversity, H_S ---Gene diversity within population, G_{ST} ---Genetic differentiation among populations

Genetic diversity with cpSSR markers

The nine variable chloroplast microsatellite primers gave 26 different cpSSR alleles among the 500 individuals surveyed. The number of alleles per loci ranged from two (AC-05) to three (AC-01, AC-03, AC-04, AC-06, AC-07, AC-08, AC-14 and AC-15) with an average of 2.8 per loci. The largest fragments amplified from the various cpSSR primer pairs were found in the range of 385 bp to 315 bp while the smallest fragments were found approximately in the range of 271–180 bp (Table 3). The cpSSR pattern with primer AC-14 is shown in Fig. 2.

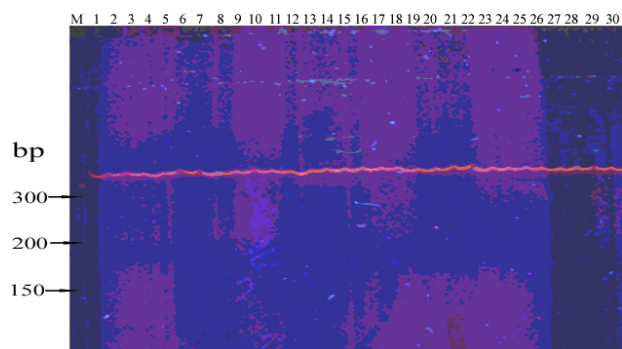


Fig. 2 cpSSR profile of *A. calamus* genotypes (Lane 1–30) generated using chloroplast microsatellite marker AC-14. Photograph showing the amplified polymorphic bands. Lane M is the molecular weight marker (low molecular weight ladder).

The averaged total gene diversity (H_T) of the nine polymorphic loci among the populations was higher (0.530 ± 0.005) in comparison to that within a population ($H_S = 0.140 \pm 0.002$). Maximum genetic diversity was found within the population A-18 (0.525) and minimum in A-25 (0.142). The mean coefficient of population differentiation (G_{ST}) among various populations was high (0.735) and the estimated gene flow (N_m) between the

populations was low (0.179) (Table 4). The genetic identity based upon the Nei's genetic similarity index ranged from the 0.58 to 0.99. The highest genetic similarity of 0.99 was found between the population A-44 and A-45 & A-51 and A-53, while the lowest genetic similarity (0.58) was found between populations A-24 & A-25, indicating high genetic divergence between them.

Analysis of molecular variance (AMOVA)

The hierarchical level of molecular divergence among populations was confirmed by the analysis of molecular variance (AMOVA). The total molecular variation was divided only two levels i.e. among populations and within population using both RAPD & cpSSR markers. The AMOVA analysis revealed significant difference ($p < 0.001$) among the populations and among individuals within a population. The ' F ' statistics i.e. Wright's F_{ST} (1978), which indicates the degree of correlation between genes drawn at different hierarchical levels of a subdivided population and estimates the differentiation among populations was 0.843 and 0.328 using RAPD and cpSSR analysis, respectively (Table 5).

Table 5. Analysis of molecular variance (AMOVA) for fifty populations of *A. calamus* using RAPD & cpSSR markers.

Source of variation	d.f.	SS	MSS	Variance Component	Variance Percentage	Fixation indices	<i>P</i> value
RAPD							
Among populations	49	2532.3	51.67	9.96 V_a	84.35	$F_{ST} = 0.843$ ***	0.000
Among individuals within population	450	369.8	1.84	1.84 V_b	15.65		
Total	499	2902.1	11.65	11.81			
cpSSR							
Among populations	49	285.33	5.82	0.82 V_a	32.86	$F_{ST} = 0.328$ ***	0.000
Among individuals within population	450	337.80	0.75	1.68 V_b	67.14		
Total	449	623.13	1.38				

d.f.- degree of freedom, SS- sum of squares, MSS- mean sum of squares; Significant at level of $p < 0.001$

Genetic structure

Genetic relationships among the populations were established using UPGMA cluster analysis, based upon the Nei's genetic similarity index. The dendrogram was developed for 50 populations of *A. calamus* using both RAPD and cpSSR markers. The genetic relationship among populations using 10 RAPD markers, were grouped into two major clusters denoted as cluster-I and cluster-II. These contained 44 and 6 populations respectively at a bootstrap value of 49%. Cluster-I could be subdivided into two sub-clusters denoted as A and B. The sub-cluster A contained 16 populations and sub-cluster B contained 28 populations. Similarly, cluster-II contained 6 populations and was divided into two sub-clusters denoted as C and D. Three populations represented in each of the sub-clusters. The cluster-II was a distinct cluster from all other clusters and was represented by the populations

from Kashmir valley except the population A-44, which was collected from the Khaziar Lake (H.P.) (Fig. 3).

The genetic relationships among the populations based upon the cpSSR markers, were grouped into two major clusters denoted as cluster-I and cluster-II. These contained 43 and 7 populations respectively at a bootstrap value of 48%. Cluster-I could be subdivided into two sub-clusters denoted as A and B. The sub-cluster A contained 15 populations and the sub-cluster B contained 28 populations. Whereas, the cluster-II contained seven populations and was divided into two sub-clusters denoted as C and D. The sub-clusters C was represented by four populations and the sub-cluster D contained three populations. The populations from Kashmir region i.e. A-51, A-52, A-57, A-58, A-59 and one population from Himachal Pradesh A-44 formed a close sub-cluster in the sub-cluster-B but here they did not form a separate cluster like in RAPDs (Fig. 4).

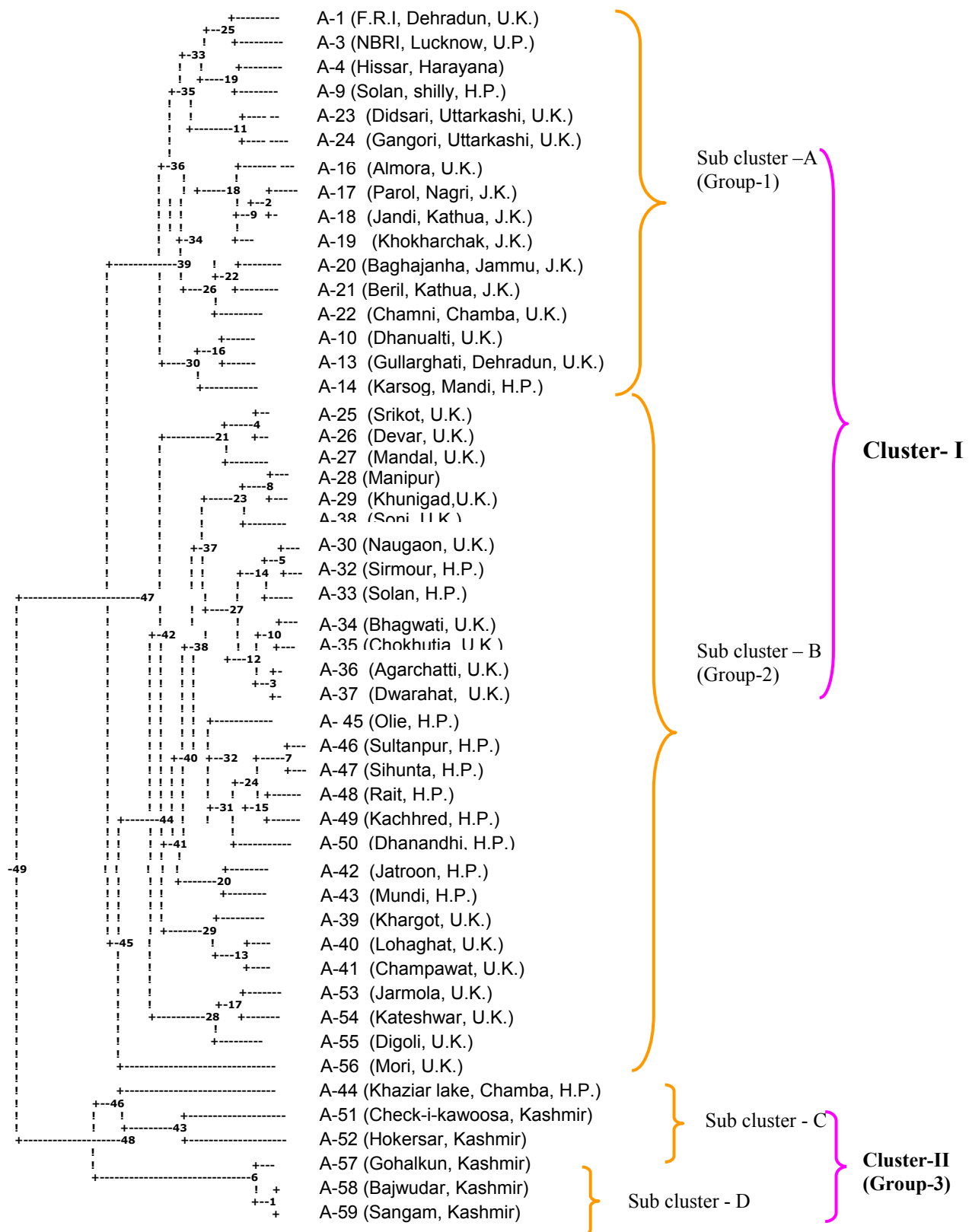


Fig. 3 Dendrogram developed using UPGMA (unweighted pair group method based on arithmetic average analysis) based on Nei's genetic similarity matrix, showing relationships between various *A. calamus* populations using ten random decamer primers. The clusters are denoted on the right side as cluster-I and cluster-II and the sub-clusters as A, B, C and D.

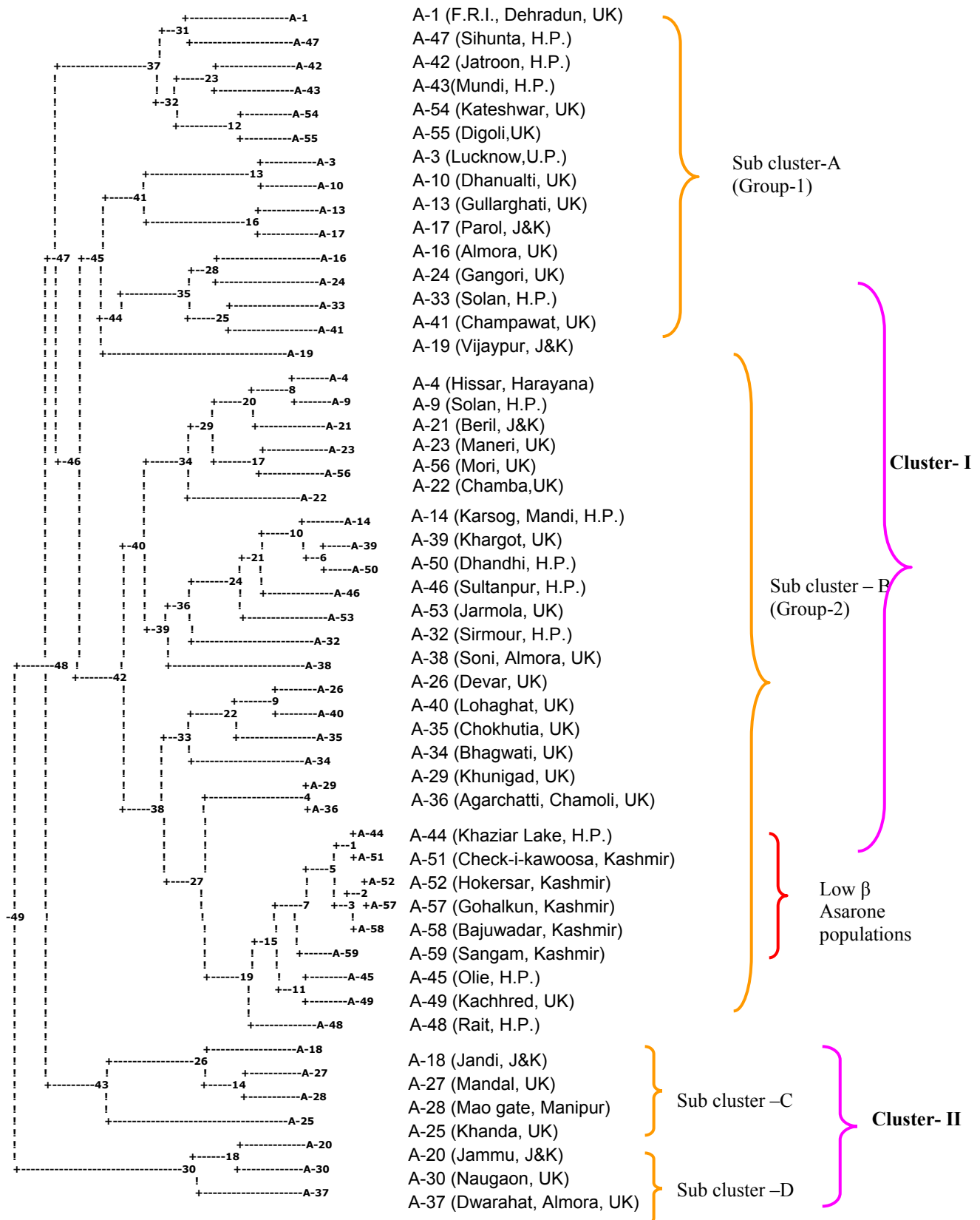


Fig. 4 Dendrogram developed using UPGMA (unweighted pair group method based on arithmetic average analysis) based on Nei's genetic similarity matrix, showing relationships between various *A. calamus* populations using nine chloroplast microsatellite primer pairs. The clusters are denoted on the right side as cluster-I and cluster-II and the sub-clusters as A, B and C, D.

Correlation analysis

The correlations, which were attempted to establish relationship between the geographical attributes of the populations (latitude, longitude and altitude) with the gene diversity, was found non significant ($p > 0.05$) as none of the geographical co-ordinates were found to have significant co-relation with the gene diversity of the population.

Discussion

The application of RAPD and cpSSR markers in genetic fingerprinting of plants, which are morphologically similar or indistinguishable, has been established as a reliable, efficient and very informative technique (Irwin et al. 1998). Despite the doubts of reproducibility with RAPDs, the technique has been yielding valuable and reproducible results which were evident recently on Piper species by Sen et al. (2010).

The investigation revealed that the overall genetic diversity within populations is very less in comparison to across the populations. Similar findings were also reported by Pai (2005) in Ohio (USA) where high molecular variation (71%) was recorded among populations of *A. calamus* than within populations i.e. only 29%. Similarly, Liao and Hsiao (1998) had reported low level of molecular variation (36.28%) within individuals of the *Acorus gramineus* collected from Western Taiwan, in comparison to the among individuals of the different sampling sites i.e. 46.84%.

A very low level of within population gene diversity indicates that the populations are considerably homogenous. Low diversity can be attributed to rare flowering & fruit setting, and restricted pollen movement. The diaspore initiating a population in *A. calamus* has been either a seed or a rhizome and subsequent growth and expansion of population patch occurs due to clonal reproduction of ramets by the rhizome (Pai 2005). No geographic variation pattern was observed in the present investigation as none of the geographical coordinates were found to have significant correlations with the gene diversity of the populations.

The overall degree of genotypic differentiation (G_{ST}) for various *A. calamus* populations was considerably high but the gene flow (N_m) across the populations was very poor. These estimates are far below than the estimates in other plant species for example *Jatropha curcas* ($N_m = 0.518$) (Gupta et al. 2008). The relatively high genetic differentiation among populations might be due to inefficient gene flow, which is the factor usually supported by cross-pollination through effective pollen transfer, seed dispersal mechanism (Lee et al. 2000) and presence of strong supporters for gene exchange (Terrab et al. 2006). Inefficiency of gene flow in *A. calamus* is influenced by the scattered distribution of the species, which may prevent or limits it, among the different groups of populations. The other factors are the strong geographical barriers due to its occurrence in mountainous areas and populations are separated by valleys, higher altitudinal zones, and rivers etc., which are responsible for the less transfer of pol-

len grains and seed dispersion. These factors are responsible for limiting the gene flow between populations and the genetic drift/or natural selection may have caused the high genetic divergence observed among plants of different populations (Pai 2005). Similar strong genetic differentiations among fragmented populations have been reported in a wind-pollinated tree, *Juniperus communis* (Provan et al. 2008).

Habitat fragmentation is responsible for generation of small isolated populations and inbreeding depression. Small isolated populations are particularly susceptible to genetic drift and inbreeding, both of which are thought to reduce genetic diversity (Frankham et al. 2002; Gaggiotti 2003). Ultimately, the species may risk extinction due to loss of genetic diversity (Wright 1943; Frankham et al. 2002). Low genetic diversity and/or inbreeding depression have been reported from many small and isolated populations, e.g., *Commiphora wightii* (Haque et al. 2009), *Oroxylum indicum* (Jayaram and Prasad 2008). Moreover, gene flow among isolated populations may reduce greatly; as a result, we may expect increased genetic differentiation among isolated populations.

The population genetic structure from RAPD cluster analysis (Fig. 1) revealed that the populations A-44, A-51, A-52, A-57, A-58 and A-59 showed the high level of genetic distinctiveness from all the other populations. These populations originate from Kashmir valley except one population i.e. A-44, which is collected from Khaziar Lake (Himachal Pradesh). This population showed the maximum genetic similarity (76.45%) with the population A-51 as revealed by the dendrogram (Fig. 1) and similarity index matrix. Whereas, the other populations i.e. A-51, A-52, A-57, A-58 and A-59 collected from Kashmir region showed 82.87% to 99.37% genetic similarity among them and the 49.90% to 55.03% genetic distinctness from all the other populations. The difference in weather pattern may partly account for the observed genetic distinctiveness of the populations of Kashmir with others.

Similarly the population genetic structure based upon the cpSSR markers also revealed genetic similarity between A-44, A-51, A-52, A-57, A-58 and A-59. The population A-44 showed the maximum genetic similarity (97.20%) with the population A-51. Whereas, the other populations i.e. A-51, A-52, A-57, A-58 and A-59 collected from Kashmir region showed 87.06% to 92.41% genetic similarity among them and 48.03% to 52.10% genetic distinctness from all the other populations.

In order to describe the distinctness of the populations A-44, A-51, A-52, A-57, A-58 and A-59 through RAPD & cpSSR, it is imperative to mention here that in another study carried out by us (unpublished) with the objectives to find out populations having low β -asarone compound in nature, the concentration of β -asarone compound and their ploidy level through chromosomal studies was checked. It was interesting to note that the populations of Kashmir valley were all hexaploids ($2n = 6x = 72$) and the one population A-44 from Khaziar lake (Himachal Pradesh) was diploid ($2n = 2x = 24$). All other populations were triploid and tetraploids. Mainly the diploid and hexaploid varieties are known to have low concentration of carcinogenic β -asarone

compound in their oil (Tomasset 1966; Mazza 1985). The dendrogram revealed this genetic relationship very clearly and grouped all hexaploids and one diploid populations in one cluster than others (Fig. 1).

The high degree of genetic divergence observed among individuals of the population A-44, A-51, A-52, A-57, A-58 and A-59 resulted partly from the fact that, some amplified RAPD and cpSSR fragments were found specific for them and these fragments were found completely absent in other populations. These markers may be useful for the future studies for identifying specific genotypes.

The morphological variation of the populations of *A. calamus* is very small (Liao and Hsiao 1998) while the genetic diversity as revealed by RAPD and cpSSR markers is comparatively high. Low morphological variation may be due to the fact the riparian habitats of plants of different populations is similar. The reproductive isolation between plants of different populations may be the main reason for high genetic divergence (Liao and Hsiao 1998) among them.

Conclusion

Conservation of plant genetic resources aims to maintain as much genetic diversity as possible. *In situ* conservation planning requires choice of populations, delimitation of sites, and continuous management and monitoring of designated populations (Iwanaga 1996; Ouédraogo 1996; Bi et al. 2003). Results indicated that genetic variability in *A. calamus* mainly resides at the interpopulation level, with low values for allelic richness, expected heterozygosity and interpopulation gene flow. Based on these results, we recommend protecting populations from as many distinctive ecological sites as possible, regardless of their size, because some distinct/rare alleles were observed in small populations. Once populations have been selected for *in situ* conservation, sound management is necessary to preserve a high level of genetic variability. The reintroduction of the threatened genotypes (genotypes having rare alleles) at regular time intervals would allow the maintenance of a substantial level of genetic variability in some endangered populations (Bi et al. 2003). The results also have implications for the *ex-situ* conservation of genetically diverse *A. calamus* plants through vegetative multiplication. The populations viz. A-44, A-51, A-52, A-57, A-58 and A-59 deserve attention in genetic conservation programs due to the fact that they are genetically distinct as well as possess the low concentration of carcinogenic β -asarone in their essential oil (unpublished). Those populations have scope to be used for large-scale multiplication for their commercial cultivation. The results indicated that there is no or very little gene flow among the populations that can be helpful for the preservation of the genetic distinctiveness of the populations.

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